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Potential Role of Fibroblast Growth Factor 21 in the Deterioration of Bone Quality in Impaired Glucose Tolerance

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Abstract:	Purpose: Findings on trabecular bone score (TBS), an index of bone quality, have been reported in prediabetes defined by impaired fasting glucose or HbA1c. Here we assessed the bone mineral density (BMD) and TBS in prediabetes individuals with impaired glucose tolerance (IGT), and investigated the association of these bone parameters with serum levels of fibroblast growth factor 21 (FGF21), a hormone implicated in bone metabolism and with higher levels in IGT. Methods: Chinese postmenopausal women aged 55–80, without diabetes, were recruited from the Hong Kong Cardiovascular Risk Factor Prevalence Study in 2016–2018. Normal glucose tolerance (NGT) was defined by fasting glucose <5.6mmol/L and 2-hour plasma glucose (2hG) <7.8mmol/L, while IGT by 2hG 7.8-11mmol/L. Serum levels of FGF21 and other bone metabolism regulators were measured. Insulin sensitivity was assessed by the Matsuda index. Independent determinants of TBS were evaluated using multivariable stepwise linear regression. Results: 173 individuals with NGT and 73 with IGT were included. TBS was lower in those with IGT compared to those with NGT, while BMD was comparable. Individuals with IGT had significantly higher serum FGF21 levels, which in turn showed an independent inverse relationship with TBS, attenuated after inclusion of the Matsuda index. Serum FGF21 levels, however, did not correlate with BMD. Conclusion: Among Chinese postmenopausal women, bone quality was worse in IGT, despite comparable bone density. FGF21 levels showed a significant independent inverse relationship with TBS, partly attributed to insulin resistance. Whether FGF21 contributes to the impaired bone quality in IGT remains speculative.
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SUMMARY

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Methods: Chinese postmenopausal women aged 55–80, without diabetes, were recruited from the Hong Kong Cardiovascular Risk Factor Prevalence Study in 2016–2018. Normal glucose tolerance (NGT) was defined by fasting glucose <5.6mmol/L and 2-hour plasma glucose (2hG) <7.8mmol/L, while IGT by 2hG 7.8-11mmol/L. Serum levels of FGF21 and other bone metabolism regulators were measured. Insulin sensitivity was assessed by the Matsuda index. Independent determinants of TBS were evaluated using multivariable stepwise linear regression.

Results: 173 individuals with NGT and 73 with IGT were included. TBS was lower in those with IGT compared to those with NGT, while BMD was comparable. Individuals with IGT had significantly higher serum FGF21 levels, which in turn showed an independent

inverse relationship with TBS, attenuated after inclusion of the Matsuda index. Serum FGF21 levels, however, did not correlate with BMD.

Conclusion: Among Chinese postmenopausal women, bone quality was worse in IGT, despite comparable bone density. FGF21 levels showed a significant independent inverse relationship with TBS, partly attributed to insulin resistance. Whether FGF21 contributes to the impaired bone quality in IGT remains speculative.

KEYWORDS: osteoporosis; bone density; prediabetes; fibroblast growth factor 21; Chinese; insulin resistance; hyperglycaemia

Introduction

Trabecular bone score (TBS) is an indirect index of bone quality which has been found to improve fracture risk assessment by clinical risk factors and bone mineral density (BMD), and has recently been incorporated into FRAX, a well-known fracture risk assessment tool [1]. Type 2 diabetes is associated with a lower TBS [2] despite a comparable or even higher BMD, compared to individuals without diabetes [3]. On the other hand, studies on TBS in prediabetes have yielded conflicting results.

Prediabetes can be identified by the presence of impaired fasting glucose (IFG; FG 5.6-6.9 mmol/L), impaired glucose tolerance (IGT; the 2-hour plasma glucose [2hG] during a 75g OGTT 7.8-11.0 mmol/L), or HbA1c 5.7–6.4% (39–47 mmol/mol) [4]. Individuals may be classified as having prediabetes by one diagnostic criterion but not by one or more of the others [5]. Notably, in a study of obese and overweight Caucasians, IGT diagnosed more individuals with prediabetes and diabetes [6]. Among studies of TBS in prediabetes, the Geelong Osteoporosis Study revealed no significant difference in the TBS between individuals with normal FG and those with prediabetes defined by IFG [7], whereas the Vietnam Osteoporosis Study revealed lower TBS values in prediabetes defined by HbA1c

5.7-6.4% compared with normal individuals among women, but not in men [8]. The difference in the results may be explained by the different diagnostic criteria for prediabetes employed. The impact of IGT on TBS has yet to be evaluated.

Circulating levels of fibroblast growth factor 21 (FGF21), an insulin-sensitizing metabolic hormone predominantly secreted from the liver and adipocytes, are raised in insulin-resistant states, including obesity and type 2 diabetes [9], which may represent a compensatory response to FGF21 resistance or metabolic changes associated with insulin resistance, such as raised free fatty acid levels [10]. Serum FGF21 levels were also reported to be higher in prediabetes, particularly in IGT [11,12]. Despite the beneficial effects on glucose and lipid metabolism of FGF21 in animals and in humans treated with FGF21 analogues [13], its potential adverse effect on bone homeostasis was a concern [14,15], in view of the enthusiasm on FGF21 as a therapeutic target for metabolic diseases [13]. Studies in humans on the association of FGF21 levels with BMD have yielded inconsistent results, ranging from a positive correlation [16,17], but no relationship to bone turnover markers or fragility fractures [17], to no correlation [18], to an inverse correlation (over hip region only) [19]. However, glycaemia and insulin resistance, parameters closely related to circulating FGF21 levels, were not explicitly

addressed in these studies. The relationship between serum FGF21 levels and TBS also has not been investigated.

Hence, we carried out this study to examine the effect of IGT on BMD and TBS in Chinese postmenopausal women, and the association of circulating FGF21 levels with BMD and TBS.

Methods

Participants

We conducted a cross-sectional case-control study of Chinese postmenopausal women aged 55 to 80 years. Participants were recruited between November 2016 and October 2018 from the Hong Kong Cardiovascular Risk Factor Prevalence Study (CRISPS) cohort. CRISPS was a long-term, community-based cohort study on cardiovascular risk factors in Hong Kong. 2900 unrelated Chinese individuals, aged between 25 and 74, were randomly recruited from the community in Hong Kong in the year 1995-1996 [20]. The cohort had been followed up prospectively to assess for the development of type 2

diabetes and other cardiovascular risk factors, during reassessment visits in 2000-2004, 2005-2008, 2010-2012, and 2016-2018. All individuals without known diabetes underwent a 75g OGTT. During the 2016-2018 visit, those without diabetes were recruited to this study.

Individuals were excluded if they (i) were already on anti-osteoporosis therapy, (ii) had secondary causes of osteoporosis, (iii) had BMI <15 or >37 kg/m^2 (when TBS measurement may not be accurate), (iv) had estimated glomerular filtration rate (eGFR) <30 mL/min, or (v) were on fibrate therapy.

The study followed the principles in the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB Ref.: UW 16-510). All participants gave informed consent.

Clinical and biochemical assessments

Participants attended a clinical assessment session after an overnight fast for at least 8 hours. Demographic data and medical history were obtained using a standardized

questionnaire. Personal and family history of fragility fractures (spine, hip, humerus, wrist and ankle) was recorded. Personal history of cardiovascular diseases, including ischaemic heart disease and stroke, was recorded. Important clinical risk factors of osteoporosis were evaluated, including smoking, drinking, family history of fragility fractures, parental history of hip fractures, prior use and duration of hormonal replacement therapy (<1 year, 1-5 years, 6-10 years, >10 years), and levels of physical activity. Daily calcium intake was assessed using a semi-quantitative questionnaire [21]. In a subgroup of the cohort, detailed dietary history was taken using a food frequency questionnaire (FFQ) with 7-day recall [22]. Using food composition tables for Hong Kong, quantification of each nutrient intake was derived by summation of the nutrients obtained from all food items in the FFQ. Body weight, body height and blood pressure (BP) were measured. Hypertension was defined as BP ≥140/90 mmHg or the use of antihypertensive medications. Fasting blood was drawn for plasma glucose, HbA1c, lipid profile, albumin, calcium, and creatinine levels. eGFR was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Dyslipidaemia was defined as fasting triglycerides (TG) ≥1.69 mmol/L, high-density lipoprotein cholesterol (HDL-C) <1.04 mmol/L in men and <1.29 mmol/L in women, low-density lipoprotein cholesterol (LDL-C) ≥3.4 mmol/L, or the use of lipid-lowering agents. Blood was stored

in aliquots at −70°C for assays of biomarkers. Serum 25-hydroxyvitamin D (25OHD) levels were measured with enzyme immunoassays (Immunodiagnostic Systems) with a sensitivity of 17.0nmol/L, and intra- and inter-assay coefficients of variation (CV) of 1.9-3.7% and 3.7-11.6% respectively. Serum FGF21 levels were measured with an enzyme-linked immunosorbent assay (ELISA) kits (Antibody and Immunoassay Services, University of Hong Kong, Hong Kong, China) [23]. The intra- and inter-assay CV of the FGF21 ELISA were 4-5% and 3.5-10.2%, respectively [24]. Serum levels of three bone metabolism regulators, osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL) and parathyroid hormone (PTH), were measured with ELISA kits (BioVendor Research and Diagnostic Products). The intra- and inter-assay CV of the OPG ELISA were 2.5-4.9% and 1.7-9.0% respectively. The intra- and inter-assay CV of the RANKL ELISA were 7.25-11.51% and 11.21-12.77% respectively. The intra- and inter-assay CV of the PTH ELISA were 1.1-2.0% and 2.9-7.1% respectively.

Definitions of glycaemic status and Matsuda index of insulin sensitivity

IGT was defined by 2hG 7.8-11.0 mmol/L during OGTT [4]. Normal glucose tolerance (NGT) was defined by FG <5.6 mmol/L and 2hG <7.8 mmol/L. Insulin sensitivity was

represented by the Matsuda index calculated by the formula

$$\frac{10000}{\sqrt{fasting\ glucose\ (mg/dL) \times fasting\ insulin (mIU/L) \times 120\ \text{min}\ glucose\ (mg/dL) \times 120\ \text{min}\ insulin\ (mIU/L)}}$$

which highly correlated with the insulin sensitivity obtained with euglycaemic clamp [25].

BMD and TBS measurements and vertebral fracture assessment (VFA)

BMD at the lumbar spine, femoral neck, and total hip, as well as VFA, were measured with a dual-energy x-ray absorptiometry (DXA) machine (Hologic QDR 4500, Waltham, MA, USA). TBS was measured with TBS iNsight™ version 3.0.2.0. BMD T-scores adjusted for TBS were calculated with the equations derived by Leslie et al [26]. All VFA images were evaluated using the Genant semi-quantitative approach according to the recommendations from the International Society for Clinical Densitometry (ISCD). Vertebral fractures were diagnosed when vertebral height was reduced by >20%, i.e., Genant Classification grade 1 or above.

FRAX and TBS-adjusted FRAX

The Hong Kong version of FRAX was used in this study, with the online tool [27]. For each

participant, four 10-year probability scores were generated: (i) major osteoporosis fracture (MOF) with BMD, (ii) MOF with BMD, adjusted for TBS ($FRAX_{adj}$); (iii) hip fracture with BMD, and (iv) hip fracture with BMD, adjusted for TBS ($FRAX_{adj}$). FRAX score ratios, both for MOF and hip fracture, were calculated as $FRAX_{adj}$/FRAX, to reflect the impact of TBS on fracture risk assessment.

Statistical analyses

Results were reported as means ± standard deviations, medians with interquartile ranges (IQR) for skewed data, or percentages as appropriate. Comparisons of clinical, laboratory, DXA-based parameters (BMD and TBS), and FRAX ratios between individuals with IGT and NGT were performed with t-test and Chi-square test as appropriate. Partial correlations between BMD/TBS and clinical and laboratory parameters were assessed, with adjustment for age and BMI. Variables which showed significant correlations with TBS were included in the subsequent multivariable model, where multivariable stepwise linear regression analysis was used to identify the independent determinants of TBS. Two-sided p-values <0.05 were considered statistically significant. All statistical analyses were performed with IBM® SPSS® Statistics version 25 for Windows.

Results

246 individuals were included in this analysis, 173 with NGT and 73 with IGT. This cohort had a mean age of 61.4±5.1 years, BMI of 24.2±3.7 kg/m^2, HbA1c of 5.6±0.3 %, and 25OHD level of 48.6±12.1 nmol/L.

We compared the BMD and TBS between individuals with NGT and those with IGT. (**Table 1**) Age was comparable between the two groups. Both hypertension and dyslipidaemia were more common in the IGT group. Clinical risk factors of osteoporosis were otherwise comparable between the two groups. Among the participants who had prior use of hormonal replacement therapy, the duration was comparable between the two groups (p for trend = 0.246). The carbohydrate and protein intake in the sub-cohort of individuals who had completed the FFQ (n=34 in NGT and n=12 in IGT) was comparable between the two groups (carbohydrate: 217 [IQR: 155-261] in NGT, vs 211 g/day [IQR: 177-261] in IGT, p=0.617; protein: 80.9 [IQR: 62.7-121] in NGT, vs 82.8 g/day [64.0-110] in IGT, p=0.635). Compared with individuals with NGT, individuals with IGT had a higher BMI (25.3±3.66 vs 23.7±3.65 kg/m^2, p=0.002), FG (5.2±0.46 vs 4.8±0.36 mmol/L,

p<0.001), HbA1c (5.8±0.33 vs 5.6±0.31 %, p<0.001), fasting insulin, and 2-hour insulin, and a lower Matsuda index. Serum FGF21 levels were higher in the group with IGT (130.2 vs 104.2 pg/mL, p=0.025). Among the bone metabolism regulators, only PTH levels were different between the two groups, being higher in IGT.

BMD was not different between the two groups at any site. TBS was lower in the group with IGT (1.27±0.08 vs 1.29±0.07, p=0.007). Comparison of the FRAX ratios between NGT and IGT groups showed that FRAX ratio (MOF) was higher in the IGT group than the NGT group (1.16±0.14 vs 1.11±0.14, p=0.016), whereas FRAX ratio (hip fracture) was not significantly different between NGT and IGT groups. BMD T-scores adjusted for TBS were not different between the groups at any site.

We evaluated the partial correlations between BMD/TBS and clinical or laboratory parameters, adjusted for age and BMI. (**Table 2**) 2hG, fasting insulin, and 2-hour insulin showed significant inverse correlations with TBS. Serum FGF21 and PTH levels inversely correlated with TBS, while the Matsuda index showed a significant positive correlation with TBS. On the other hand, none of the metabolic and biochemical parameters, nor serum FGF21 levels correlated with BMD at any site, except OPG which positively

correlated with LS BMD. Nonetheless, both serum OPG levels and BMD were comparable between NGT and IGT groups. TBS was lower among individuals with hypertension than those without (1.27±0.07 vs 1.29±0.07, p=0.028), while TBS was comparable between individuals with and without dyslipidaemia (1.28 ± 0.07 vs 1.29±0.07, p=0.159).

We further examined the independent determinants of TBS in the multivariable linear regression analyses. (**Table 3**) In **Model 1** which included clinical variables (age, BMI, hypertension and IGT) and FGF21, the IGT state remained an independent determinant of TBS, in addition to age. Moreover, serum FGF21 levels showed an independent inverse correlation with TBS. In the subsequent **Model 2**, we examined the potential mechanisms mediating the relationship between serum FGF21 levels and TBS. Hence, we further included the Matsuda index (the insulin sensitivity index) and PTH (the bone metabolism regulator) into the model. In this final model, only age, serum FGF21 levels and the Matsuda index remained significant, while PTH was not significant (p=0.086). Although inclusion of the Matsuda index into the model attenuated the correlation between serum FGF21 levels and TBS, serum FGF21 levels remained independently and inversely associated with TBS.

Discussion

Our study revealed that bone quality was worse in IGT despite comparable bone density among Chinese postmenopausal women, which added to the existing findings regarding TBS in prediabetes. Furthermore, we reported for the first time an independent inverse relationship between serum FGF21 levels and TBS, suggesting a potential role of FGF21 in the deterioration of bone quality in IGT.

Two studies have addressed the association between prediabetes and TBS. The Geelong Osteoporosis Study found no difference in TBS between individuals with normal FG and those with IFG [7], while the Vietnam Osteoporosis Study demonstrated a lower TBS among those with HbA1c 5.7-6.4% compared with those with normal HbA1c only in women [8]. Given the advantage in our cohort that every participant had an OGTT, we were able to demonstrate specifically the presence of reduced TBS values among the individuals with IGT, compared to those with NGT, thus expanding the current understanding on TBS changes in prediabetes. As the HbA1c approaches normal levels, postprandial glucose levels contribute more to the HbA1c [28]. Thus, our findings are also in line with those of the Vietnam Osteoporosis Study, and in fact, suggest that the

deterioration in bone quality in prediabetes may be particularly relevant in the IGT state. The postulated mechanism for the association between lower TBS values and IGT is the accumulation of advanced glycation end-products (AGEs) associated with chronic hyperglycaemia [29]. The difference in TBS values between IGT and NGT groups is of potential clinical relevance since we demonstrated a higher FRAX ratio for MOF in the IGT group than the NGT group. This finding suggested that TBS adjustment increased the FRAX scores in individuals with IGT to a greater extent than NGT, which in turn may translate to a more accurate reflection of the fracture risks in different glycaemic status.

Our study generated an interesting finding of an inverse correlation between serum FGF21 levels and TBS, independent of age and BMI, the well-described clinical factors affecting TBS [30]. Further inclusion of the Matsuda index into the multivariable linear regression model attenuated the association between FGF21 levels and TBS, which remained significant. These findings suggested that the inverse relationship between serum FGF21 and TBS may in part be attributed to the high FGF21 levels in insulin resistance [11], an established risk factor of reduced TBS [31,32]. Indeed, our study demonstrated a positive independent relationship between the Matsuda index of insulin sensitivity and TBS, suggesting an adverse effect of insulin resistance on the bone

microarchitecture consistent with previous studies [31,32]. In a Korean study, including individuals with and without diabetes, HOMA-IR inversely correlated with TBS in the age- and BMI-adjusted model [32]. Similar findings were reported for non-diabetes individuals in the FORMEN study which included men only. In the FORMEN study, the inclusion of bone turnover markers and pentosidine levels into the model did not change the association of TBS with HOMA-IR. Hence, it was postulated that hyperglycaemia in the insulin-resistant state could lead to the deterioration in bone microarchitecture through mechanisms other than bone turnover and AGEs [31]. Our findings showed that the inverse relationship between insulin resistance and bone quality applied to non-diabetes women as well.

The inverse correlation between serum FGF21 levels and TBS, independent of age, BMI, IGT state, insulin resistance and PTH suggested a direct adverse effect of FGF21 on bone. The potential pathophysiology has been elucidated in studies on rodents. High expression levels of FGF21 in mouse liver induced the secretion of IGFBP1, which bound with integrin beta-1 on osteoclast precursors, potentiated RANKL-stimulated Ark-phosphorylation and NFATc1 activation and consequently promoted osteoclastogenesis and bone loss [15]. FGF21 may also promote bone loss and potentiate the adverse bone

effect of PPAR-gamma agonists in mice [14], although this was not replicated in a more recent study [33]. In humans, serum FGF21 levels were shown to be associated with a worsened radial trabecular bone microarchitecture and decreased radial bone strength among women with anorexia nervosa, using high-resolution peripheral quantitative computed tomography (HRpQCT) [34], suggesting that FGF21 may play a role in the deterioration of bone quality in clinical conditions without increased insulin resistance, through alternative mechanisms. All these preclinical and clinical studies supported the deleterious role of FGF21 on bone. The absence of significant correlations between serum FGF21 levels and BMD in our study may suggest that the FGF21 affects the bone quality more than the bone density in prediabetes individuals and thus a significant effect of FGF21 on the bone may not be observed on BMD measurements.

The finding of an inverse correlation between serum FGF21 levels and TBS is of clinical relevance and importance in view of on-going clinical trials on FGF21 agonists in the treatment of metabolic disorders characterized by insulin resistance such as non-alcoholic fatty liver disease (NAFLD) and diabetes [13]. Furthermore, the metabolic benefits of co-agonists of glucagon-like peptide 1 (GLP-1) and glucagon receptors, another new class of medications being explored for the treatment of diabetes and

related metabolic diseases, were mediated in part through the potentiation of FGF21 secretion [35,36]. Two FGF21 agonists, pegbelfermin and an IgG1-Fc-fused FGF21, are currently under investigations for the treatment of NAFLD [37]. In a phase 2 study with pegbelfermin, BMD was not reduced in the pegbelfermin-treated participants at up to 6 months [38]. Our findings would call for the need to evaluate the effect of these FGF-21 based therapies on both bone density and quality.

Our study had the strength that every participant underwent a 75-gram OGTT to allow the evaluation of the differences in BMD and TBS between individuals with NGT and IGT. Furthermore, we were able to study the relationship between serum FGF21 levels and bone density and quality in an exclusively non-diabetes cohort. However, our study was cross-sectional, which allowed the demonstration of associations but not causal relationships. Besides, the bone quality was indirectly assessed with TBS. Lastly, the sample size of the cohort was relatively small to study the difference in fracture events between individuals with different glycaemic status. Hence, although there was a statistically significant difference in TBS between NGT and IGT, the TBS values of both groups belonged to the range of 'partially degraded microarchitecture' (TBS ≤1.2, 1.20-1.35 and ≥1.35 defining degraded, partially degraded, and normal microarchitecture,

respectively) [39], whether it is clinically relevant warrants further studies.

In conclusion, among Chinese postmenopausal women, the bone quality was worse in IGT despite a comparable bone density. Serum FGF21 levels showed a significant independent inverse association with TBS, which could be partly attributed to the effect of insulin resistance on circulating FGF21 levels. Whether FGF21 plays any causal role on the deterioration of the bone microarchitecture in individuals with IGT remains to be further investigated and should receive attention when developing FGF21-based therapeutics.

AUTHORS' CONTRIBUTIONS

D.T.W.L. researched the data and wrote the manuscript. C.H.L., J.K.Y.L. and A.C.H.L. researched the data. V.W.K.C., C.H.Y.F. and K.M.Y.Y. performed statistical analyses. W.S.C., K.C.B.T., Y.C.W. and K.S.L.L. critically reviewed and edited the manuscript. Y.C.W. and K.S.L.L. initiated and supervised the study, are the guarantors of this work and as such had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICTS OF INTEREST

All authors declare that they have no competing interest.

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TABLES

Table 1 Baseline characteristics of the participants

	NGT	IGT	p-value
Number (%)	173 (70.3)	73 (29.7)	—
Age, years	61.2±5.05	62.0±5.35	0.282
Ever smoker, %	4 (2.3)	3 (4.1)	0.439
Ever drinker, %	146 (84.4)	58 (79.5)	0.347
Hypertension, %	48 (27.7)	38 (52.1)	**<0.001**
Dyslipidaemia, %	95 (54.9)	53 (72.6)	**0.010**
Cardiovascular diseases (ischaemic heart disease and/or stroke), %	11 (6.4)	4 (5.5)	0.792
Hormonal replacement therapy, %	11 (6.4)	3 (4.1)	0.487
Personal history of fragility fracture, %	15 (8.7)	10 (13.7)	0.233
Personal history of vertebral fracture, %	7 (4.0)	7 (9.6)	0.086
Family history of fragility fracture, %	27 (15.6)	8 (11.0)	0.340
Parental history of hip fracture, %	14 (8.1)	4 (5.5)	0.472
Physical Activity			0.274*
Inactive, %	79 (45.7)	37 (50.7)	
Low, %	38 (22.0)	18 (24.7)	
Medium, %	31 (17.9)	11 (15.1)	
High, %	25 (14.5)	7 (9.6)	
Body mass index, kg/m^2	23.7±3.65	25.3±3.66	**0.002**
Fasting glucose, mmol/L	4.8±0.36	5.2±0.46	**<0.001**
2-hour glucose, mmol/L	5.8±1.06	9.0±0.88	**<0.001**
HbA1c, %	5.6±0.31	5.8±0.33	**<0.001**
Fasting insulin[a], mIU/L	6.1 (4.4-8.0)	8.9 (6.1-12.0)	**<0.001**
2-hour insulin[a], mIU/L	48.0 (32.4-64.5)	90.0 (64.5-130.5)	**<0.001**
eGFR, mL/min	90.0±10.90	90.9±10.51	0.543
Albumin-corrected calcium, mmol/L	2.3±0.06	2.3±0.06	0.863
25(OH)D, nmol/L	49.2±11.61	47.1±13.20	0.216
Matsuda index[a]	6.4 (4.81-8.99)	2.8 (2.16-3.69)	**<0.001**
FGF21[a], pg/mL	104.2 (54.5-165.9)	130.2 (71.9-197.1)	**0.025**
OPG[a], pmol/L	5.7 (4.99-6.50)	5.9 (5.01-6.62)	0.532
PTH[a], pg/mL	36.1 (19.13-51.73)	39.7 (25.75-67.54)	**0.007**
RANKL[a], pmol/L	178.7 (99.58-301.57)	201.6 (111.00-353.51)	0.779

BMD lumbar spine, g/cm^2	0.85±0.12	0.86±0.12	0.631
BMD femoral neck, g/cm^2	0.65±0.09	0.66±0.11	0.837
BMD total hip, g/cm^2	0.85±0.10	0.87±0.12	0.366
Trabecular bone score	1.29±0.07	1.27±0.08	**0.007**
FRAX ratio (MOF)	1.11±0.14	1.16±0.14	**0.016**
FRAX ratio (hip fracture)	1.05±0.22	1.08±0.24	0.318
BMD T-score adjusted for TBS			
Lumbar spine	-1.57±1.34	-1.70±1.41	0.483
Femoral neck	-1.41±1.01	-1.43±1.19	0.784
Total hip	-0.21±1.08	-0.18±1.25	0.838

Data presented as mean ± standard deviation, median (25th – 75th percentile) or percentages as appropriate

[a] log-transformed before analysis

*p for trend

Abbreviations: NGT, normal glucose tolerance; IGT, impaired glucose tolerance; eGFR, estimated glomerular filtration rate; 25(OH)D, 25-hydroxyvitamin D; FGF21, fibroblast growth factor 21; BMD, bone mineral density; MOF, major osteoporosis fracture; OPG, osteoprotegerin; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor kappa-B ligand; TBS, trabecular bone score

In both groups, the proportions of participants using thiazide diuretics, loop diuretics, statins, beta-blockers, and nitrates were comparable

Table 2 Partial correlation of bone mineral density and trabecular bone score with clinical and laboratory parameters, adjusted for age and body mass index

	TBS		BMD lumbar spine		BMD femoral neck		BMD total hip	
	Adjusted r	**p-value** [b]	**Adjusted r**	**p-value** [b]	**Adjusted r**	**p-value** [b]	**Adjusted r**	**p-value** [b]
Fasting glucose (mmol/L)	-0.06	0.348	-0.10	0.876	-0.04	0.515	-0.01	0.882
2-hour glucose (mmol/L)	**-0.17**	**0.008**	-0.60	0.349	-0.07	0.305	-0.02	0.766
HbA1c (%)	-0.05	0.459	0.01	0.910	0.03	0.618	0.09	0.166
Fasting insulin[a] (mIU/L)	**-0.15**	**0.016**	-0.06	0.373	-0.07	0.266	0.02	0.715
2-hour insulin[a] (mIU/L)	**-0.21**	**0.001**	-0.05	0.467	-0.04	0.498	0.06	0.346
Albumin-corrected calcium (mmol/L)	-0.03	0.645	-0.003	0.960	-0.004	0.952	0.003	0.966
eGFR (mL/min)	0.04	0.519	-0.03	0.702	0.06	0.371	0.04	0.590
25(OH)D (nmol/L)	0.07	0.283	0.02	0.794	-0.03	0.599	-0.04	0.589
FGF21[a] (pg/mL)	**-0.15**	**0.023**	-0.05	0.416	-0.10	0.107	-0.06	0.356
Matsuda index[a]	**0.22**	**0.001**	0.06	0.367	0.06	0.316	-0.04	0.512
OPG[a] (pmol/L)	-0.01	0.845	**0.15**	**0.020**	-0.04	0.507	-0.01	0.854
PTH[a] (pg/mL)	**-0.15**	**0.022**	-0.09	0.156	-0.06	0.339	-0.05	0.480
RANKL[a] (pmol/L)	0.06	0.371	-0.01	0.927	0.09	0.171	0.06	0.354

[a] log-transformed before analysis; [b] Age and BMI adjusted p-value

Abbreviations: TBS, trabecular bone score; BMD, bone mineral density; BMI, body mass index; eGFR, estimated glomerular filtration rate; 25(OH)D, 25-hydroxyvitamin D; FGF21, fibroblast growth factor 21; OPG, osteoprotegerin; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor kappa-B ligand

Table 3 Multivariable stepwise linear regression models showing the independent determinants of the trabecular bone score

	Model 1		Model 2	
	Standardized beta (95% CI)	p-value	Standardized beta (95% CI)	p-value
Age, years	**-0.16 (-0.29, -0.04)**	**0.009**	**-0.16 (-0.28, -0.04)**	**0.011**
Impaired glucose tolerance (Yes/No)	**-0.31 (-0.58, -0.04)**	**0.025**	-0.04 (-0.36, 0.29)	0.833
FGF21[a], pg/mL	**-0.14 (-0.26, -0.02)**	**0.027**	**-0.13 (-0.25, -0.004)**	**0.044**
Matsuda index[a]	—	—	**0.22 (0.07, 0.37)**	**0.004**

[a] log-transformed before analysis

Model 1: age, body mass index, hypertension, impaired glucose tolerance and FGF21

Model 2: Model 1 + (Matsuda index + parathyroid hormone)

Abbreviation: CI, confidence interval; FGF21, fibroblast growth factor 21

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Responses to Reviewers' Comments

REVIEWER #1

Main limitations

1. Have you evaluated the personal history of fractures (in particular, vertebral fractures)? TBS could be affected by the presence of fractures.

*We have evaluated the personal history of fractures among NGT and IGT groups (Page 31, **Table 1**). No significant difference was observed between the groups for personal history of all fragility fractures as well as morphometric evidence of vertebral fractures. TBS was not significantly different between those with history of any fragility fractures and those without (1.28±0.09 vs 1.29±0.07, p=0.702). Specifically, there was no significant difference in TBS between those with and without vertebral fractures (1.26±0.08 vs 1.29±0.07, p=0.133).*

2. Did the authors exclude all the variables that may potentially affected the bone quality (family history of fragility fractures, cigarettes, drugs…)?

*We have evaluated a variety of important clinical risk factors of osteoporosis, including smoking, drinking, family history of fragility fractures, parental history of hip fractures, prior use of hormonal replacement therapy and levels of physical activity. These variables were comparable between NGT and IGT groups. (Page 31, **Table 1**)*

3. Recent evidence confirmed that "Circulating FGF21 levels are robustly increased by diets that are high in carbohydrate but low in protein" (Hill Cm et al. Endocrinology. 2020).

What kind of diet were the study population followed immediately before the blood sampling?

Thank you for your insightful comment. Participants in our study were recruited from the 2016-2018 reassessment visit in CRISPS. 273 women in CRISPS had their detailed dietary history taken using a food frequency questionnaire (FFQ) with 7-day recall. Using food composition tables for Hong Kong, quantification of each nutrient intake was derived by summation of the nutrients obtained from all food items in the FFQ. Among the 273 women, 46 were included in our study. The following table showed that the carbohydrate and protein intake was comparable between the 46 participants and the 227 women not included in our study.

	Women who completed FFQ in CRISPS	**Participants of our study**	**Women not included in our study**	**p-value**	**Age-adjusted p-value**
Number	273	46	227	—	—
Age (years)	63.6±9.73	59.4±4.86	64.5±10.2	0.006	—
Carbohydrate intake (g/day)	209±76.9	230±87.1	205±73.9	—	0.076
Protein intake (g/day)	79.9±35.6	81.8±31.5	79.5±36.4	—	0.916

Among the participants of our study who have completed the FFQ, there was no significant difference in the carbohydrate and protein intake between the NGT and IGT groups, tabulated below. (Page 14, lines 227-231)

	All	**NGT**	**IGT**	**p-value**
Number of participants (%)	46 (100%)	34 (73.9%)	12 (26.1%)	—
Carbohydrate intake (g/day)	216 (167-261)	217 (155-261)	211 (177-261)	0.617
Protein intake (g/day)	80.9 (63.3-118)	80.9 (62.7-121)	82.8 (64.0-110)	0.635
FGF21 (pg/mL)	105 (67.8-189)	105 (75.5-168)	97.8 (63.9-252)	0.999

Among these 46 participants, we demonstrated, and summarized below, that circulating

FGF21 levels tended to be positively correlated with carbohydrate intake and inversely correlated with protein intake, consistent with the findings reported by Hill et al., although both did not reach statistical significance given the small sample size of this subcohort.

Spearman correlation between circulating FGF21 levels and carbohydrate/protein intake adjusted for age (n=46)

	Correlation coefficient	Age-adjusted p-value
Carbohydrate intake	0.270	0.073
Protein intake	-0.256	0.089

*As mentioned in the **Methods (Clinical and biochemical assessments)**, each participant attended the clinical assessment session for blood taking after an overnight fast for at least 8 hours. (Page 8, lines 132-133)*

4. The authors should adequately comment that even they reported a statistically significant difference in terms of TBS between NGT and IGT, both groups belong to the "partially degraded microarchitecture group" (TBS < 1.2 = degraded microarchitecture; TBS > 1.34 = "normal"). Therefore a question raises: is this difference clinically relevant?

Thank you for your comment. Although there was a statistically significant difference in TBS between NGT and IGT, due to the limited sample size, whether it is clinically relevant warrants further studies. This has been included as a limitation in the discussion. (Page 20, lines 343-348)

Minor points

1. Please show the T score BMD adjusted for TBS in table 1

*Thank you for your suggestion. We have computed the BMD T-score adjusted for TBS, using the equations derived by Leslie WD et al. (Osteoporos Int. 2018;29(3):751–8), as shown in **Table 1**. (Page 32)*

REVIEWER #2

1. Did the Authors evaluate other potential fracture risk, such as previous fractures, familiarity, physical activity, smoking habits?

   *We have evaluated a variety of important clinical risk factors of osteoporosis, including smoking, drinking, personal and family history of fragility fractures, parental history of hip fractures, prior use of hormonal replacement therapy and levels of physical activity. These variables were comparable between NGT and IGT groups. (Page 31, **Table 1**)*

2. Since a postmenopausal female population has been evaluated, did the Authors evaluate whether, and in case for how long, hormone replacement therapy had been taken by the patients evaluated in the study?

   *We have included the proportion of participants with history of hormonal replacement therapy in **Table 1**, which was comparable between NGT and IGT groups. (Page 31) The duration of hormonal replacement therapy was also comparable (p for trend = 0.246). (Page 14, lines 225-227)*

3. Since women were recruited from the Hong Kong Cardiovascular Risk Factor Prevalence Study, did they have any specific cardiovascular factor risks? It is now believed that

correlation between bone loss and cardiovascular risk might be mediated by vascular calcification. Did the Authors evaluate any other factors potentially involved in alteration of bone quality, such as bone morphogenetic proteins, osteoprotegerin, receptor activator of nuclear factor κB ligand, parathyroid hormone, oxidized lipids beside FGF21? This factor by itself might not explain alteration of bone quality in these subjects.

*Cardiovascular risk factors were evaluated among our participants with the proportions in NGT and IGT groups tabulated in **Table 1**. (Page 31) Hypertension and dyslipidaemia were more prevalent among IGT group compared with NGT, while the proportion of participants with cardiovascular diseases (ischaemic heart disease and/or stroke) was comparable. TBS was lower among participants with hypertension compared with those without (1.27±0.07 vs 1.29±0.07, p=0.028), with hypertension being a component of metabolic syndrome in which insulin resistance is the key pathophysiology. On the other hand, TBS was comparable between participants with dyslipidaemia and those without (1.28 ± 0.07 vs 1.29±0.07, p=0.159). (Pages 14-15, lines 240-242)*

*Following your advice, we have also evaluated the factors potentially involved in alteration of bone quality. Given the constraints of the available blood samples, we evaluated three of the five suggested bone metabolism regulators more commonly reported to be implicated in diabetic bone disease, osteoprotegerin [OPG], receptor activator of nuclear factor kappa-B ligand [RANKL] and parathyroid hormone [PTH]. The results were included in **Table 1**. (Page 31) While OPG and RANKL levels were comparable between NGT and IGT, PTH levels were higher in IGT than NGT (p=0.007). Among the three bone metabolism regulators, only PTH correlated with TBS (adjusted r = -0.15, p=0.022), adjusted for age and body mass index. (Page 33, **Table 2**)*

*In the multivariable stepwise linear regression analyses, circulating FGF21 levels remained significantly inversely correlated with TBS independent of IGT and insulin resistance, while hypertension and PTH were not independent determinants of TBS. (Page 34, **Table 3**)*

4. Authors stated “Whether FGF21 contributes to the impaired bone quality in IGT remains speculative”, thus title should partially modify.

Thank you for your comment. The title has been modified accordingly. (Page 1, line 1)